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Long-chain non-coding RNA LINC01554 promotes NGFR expression and inhibits cell proliferation, migration, and invasion in hepatocellular carcinoma by binding to microRNA-3681-3p

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Abstract. – OBJECTIVE: The aim of this study was to analyze the role of LINC01554 in the pathogenesis of hepatocellular carcinoma (HCC) and explore the potential mechanism through which LINC01554 affects the migration and proliferation of HCC cells.

PATIENTS AND METHODS: LINC01554 expression in HCC tissues and its link to the prognosis of patients were analyzed by The Cancer Genome Atlas (TCGA) database. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was carried out to examine LINC01554 levels in 60 cases of HCC clinical tissues and HCC cell lines. Then, LINC01554 overexpression model was constructed using lentivirus in HCC cell lines. HCC proliferation and invasive ability were evaluated through Cell Counting Kit (CCK-8) and transwell tests, respectively. Furthermore, the potential action mechanism of LINC01554 was explored using bioinformatics analysis and in vitro cell experiments.

RESULTS: Analysis of the TCGA database revealed that LINC01554 was remarkably underexpressed in HCC tissues. Decreased expression of LINC01554 predicted a poor prognosis for patients. Besides, LINC01554 overexpression markedly blunted the proliferation and migratory capacities of HCC cells. LINC01554 competed with NGFR to bind to microRNA-3681-3p, thereby providing possible mechanisms by which LINC01554 could participate in the progression of HCC.

CONCLUSIONS: This study shows for the first time that LINC01554 modulates NGFR expression by binding to microRNA-3681-3p, thereby participating in the progression of HCC.

Key Words:
HCC, LINC01554, Proliferation, Cell migration.

Introduction

Liver cancer mainly includes primary liver cancer and secondary metastatic liver cancer, among which primary liver cancer ranks fourth in the incidence of malignant tumors in the world[1]. Primary liver cancer includes hepatocellular carcinoma (HCC), primary bile duct cell carcinoma, and primary liver cells. There are three pathological types of mixed HCC of bile duct cells, among which HCC accounts for more than two-thirds, with a high incidence and mortality[2]. The main causes of liver cancer include viral hepatitis B and hepatitis C, long-term alcohol abuse, aflatoxin intake, and non-alcoholic fatty liver disease. However, the risk factors are also significantly different in different regions. Surgical resection, liver transplantation, and percutaneous radiofrequency ablation for HCC are the main treatment methods for early HCC[3,4]. Due to lack of evident clinical manifestations, most patients are already in the middle and late stage and thus lose the optimal treatment peri-

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od. Despite the advancement of medical level and the diagnosis method of liver cancer, the overall therapy efficacy remains poor, with the 5-year survival rate still lower than 20%[5]. The main reason for the high degree of malignancy and poor therapeutic effect of HCC is that HCC pathogenesis is still unclear[6]. Thus, the exploration of the pathogenesis of HCC is expected to provide new ideas for the treatment of HCC.

Long non-coding RNA (lncRNA) refers to a class of non-coding RNA transcripts which, used to be considered as "junk sequences", have attracted more attention from scholars for their important biological functions[7]. Klec et al[8] have shown significant differences in the expression levels of certain lncRNAs in normal tissues and tumor tissues, which are implicated in tumor staging and prognosis. Currently, lncRNA-related studies have become the focus of tumor molecular biology. Notably, NR2F1-AS1 promotes the malignant progression of osteosarcoma *via* enhancing the expression of forkhead box A1 through adsorption of microRNA-483-3p[9]. SNHG7 promotes cell migration and epithelial to mesenchymal transformation of NSCLCs by modulating the microRNA-449a/TGIF2 axis[10]. DLX6-AS1 accelerates the development of neuroblastoma through the regulation of microRNA-107/BDNF pathway[11].

The interaction between lncRNAs and miRNAs plays a vital regulatory role in tumors, on the basis of which the competing endogenous RNA (ceRNA) hypothesis has been proposed and confirmed by many scholars[12]. CeRNA is not a kind of newly discovered RNA, but an RNA interaction adjustment mechanism. According to this mechanism, lncRNA, miRNA, mRNA, and other RNAs are functionally regulated by each other, and once the expression of one or several of them is abnormally upregulated or downregulated, the balance between them will be destroyed, leading to related diseases. As a new hypothesis, lncRNA-miRNA-mRNA ceRNA regulatory network has attracted more and more attention[13].

In this research, The Cancer Genome Atlas (TCGA) database revealed a low expression of LINC01544 in HCC. The potential molecular mechanism of LINC01544 in HCC cell biological functions were preliminarily discussed. The novel findings provide a concept that LINC01544 may be a major regulator mediate HCC progression.

Patients and Methods

GEPIA Database

GEPIA database (http://GEPIA.cancer-pku.cn/index.html) was used to evaluate the link between LINC01544 and prognosis of HCC patients. The GEPIA online database was used to mine and analyze TCGA and GTEx gene sequencing data.

Patients and Sample Collection

The patients included in this study signed informed consent. 60 pairs of HCC tumor specimens and adjacent specimens were provided by HCC patients at The Second Affiliated Hospital of Nantong University, the First People's Hospital of Nantong City. All specimens have been confirmed by pathological examination as HCC. Inclusion criteria: patients underwent no post-operative radiotherapy and chemotherapy. Exclusion criteria: patients complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases. This study was approved by the Ethics Committee of The Second Affiliated Hospital of Nantong University, the First People's Hospital of Nantong City. All patients provided written informed consent. This investigation was conducted in accordance with the Declaration of Helsinki.

Cell Culture

Normal hepatic cell line LO2 and HCC cell lines Hep3B, Huh7, SK-Hep1 and SMMC-7721 used in the experiments were provided by America Type Culture Collection (ATCC; Manassas, VA; USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% streptomycin and penicillin in a humid atmosphere at 37°C and 5% CO_2.

Cell Transfection

Overexpression plasmid containing LINC01554 was constructed into the pSicoR lentiviral vector, and 293T cells were used for virus production. HCC cells were infected with lentivirus and selected for two weeks using puromycin to generate a cell line that was overexpressed with LINC01554. For transient transfection, Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) was mixed with miRNA or overexpression/reporter plasmids (GenePharma, Shanghai, China).

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) reagent, and 0.5 μg of total RNAs were amplified by PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan), and then, the product was subjected to qRT-PCR analysis on ABI7900 instrument with SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan) reagent. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal parameters. The primer sequences were shown below: LINC01554 Forward (5′-3′): -GAGGGCAAAAGACTGCAAGC and Reverse (5′-3′): CTCATCAACCGACCTCCCTG, microRNA-3681-3p Forward (5′-3′): GGCGTCGTACAGTGAGT and Reverse (5′-3′): CACTGCTTGTCGTGGAGT, NGFR Forward (5′-3′): CCTACGGCTACTACCAGGATG and Reverse (5′-3′): CACACGGTGTTCTGCTTGT, GAPDH Forward (5′-3′): CTCCTCCACCTTTGACGCTG and Reverse (5′-3′): TCCTCTCTTG, and U6 Forward (5′-3′): GCTGAGGTGACGGTCTCAAA and Reverse (5′-3′): GCCTCCCAGTTTCATGGACA. The relative expression of the genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Proliferation Detection

Cell Counting Kit (CCK-8) test: the cells were seeded at a concentration of 4×10^3/well in a 96-well plate. After incubated for 0, 24, 48, and 72 hours, 10 μL of CCK-8 solution was added and the incubation was continued at 37°C for 2 hours. The absorbance was then analyzed at 450 nm and the data were recorded.

Clone formation experiment: cells were seeded in a 6-well plate at 4×10^2 per well. Complete medium was used for culture, and after 14 days, visible colonies were stained with crystal violet. Finally, the stained colonies were photographed and counted.

Transwell Assay

Matrigel invasive transwell (BD Biosciences, Franklin Lakes, NJ, USA) was used for cell migration detection.

RNA Immunoprecipitation Experiment (RIP)

EZ-Magna RIP kit (Millipore, Billerica, MA, USA) was used for RIP experiments according to the instructions.

Luciferase Assay

Wild-type and mutant LINC01554 or NGFR were cloned into the pGL3-control vector. The Luciferase activity was measured using a Dual-Luciferase reporter kit (Promega, Madison, WI, USA).

Statistical Analysis

SPSS 20.0 software (SPSS IBM Corp., Armonk, NY USA) was used for statistical analysis. All results were expressed as the mean ± SD (Standard Deviation) of three independent replicate experiments. The *t*-test was used for analyzing measurement data. Differences between two groups were analyzed using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ represented a statistical difference.

Results

LINC01554 Was Downregulated In HCC Tissues and Cells

First, LINC01554 expression in HCC was analyzed through the TCGA database, and it was found to be remarkably reduced (Figure 1A). Consistent with the results, qRT-PCR analysis detected a significant reduction in LINC01554 expression in HCC tissue samples collected from 60 cases and in HCC cell lines (Figure 1B, 1C). These results indicate that LINC01554 may have important biological functions in HCC. Then, the patients were divided into LINC01554 high-expression group and low-expression group, and a markedly reduced overall survival rate of patients was found in the latter (HR = 0.51, $p<0.001$) (Figure 1D), but no significant difference in disease-free survival was observed (Figure 1E).

Overexpression of LINC01554 Attenuated the Proliferation and Invasion Abilities of HCC Cells

To test the impacts of LINC01554 on HCC cell functions, the LINC01554 overexpression plasmid and lentiviral vector were transfected in Hep3B and Huh7, and LINC01554 expression was detected to be enhanced by qRT-PCR (Figure 2A). Then, the cell proliferation was tested through CCK-8 assay and cell clone formation experiment. It was found that overexpression of LINC01554 remarkably inhibited the prolifera-

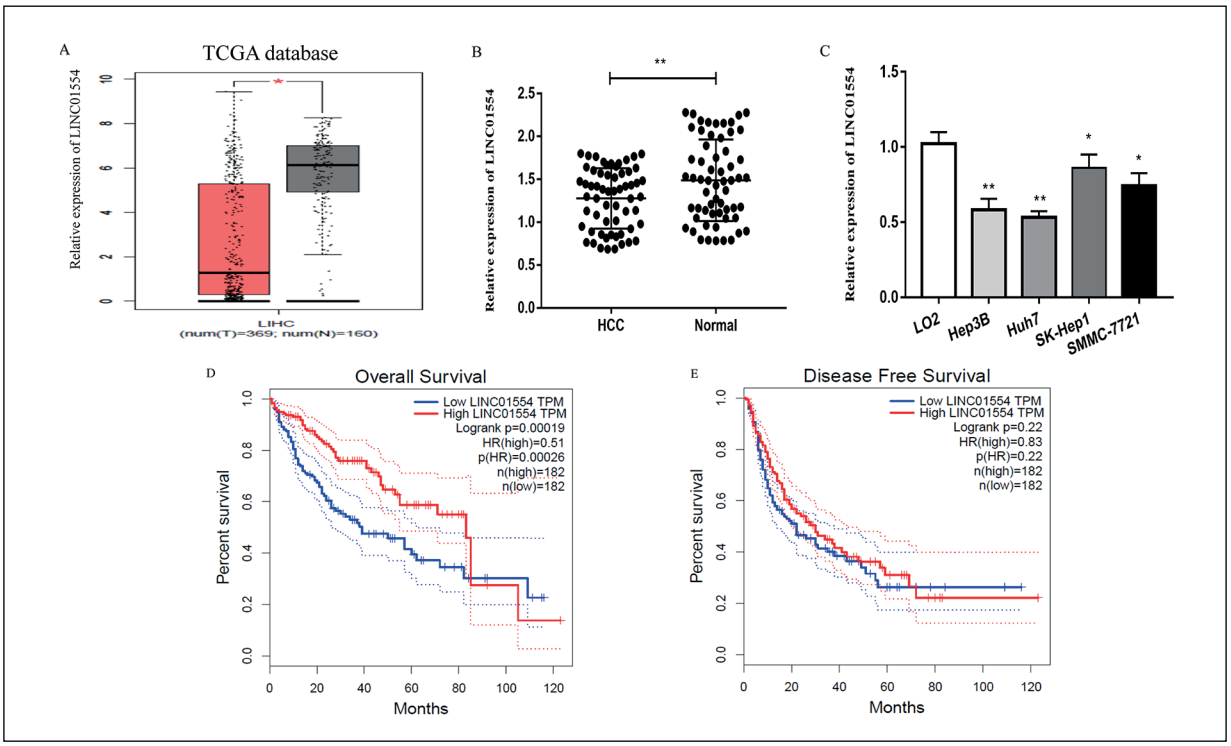

Figure 1. LINC01554 expression in hepatocellular carcinoma tissues and cell lines. **A,** LINC01554 expression level in The Cancer Genome Atlas (TCGA) database. **B,** LINC01554 expression level in hepatocellular carcinoma tissues and adjacent normal tissues. **C,** LINC01554 expression level in hepatocellular carcinoma cell lines. **D,** The relationship between the expression of LINC01554 and overall Survival of patients with hepatocellular carcinoma was analyzed by the TCGA database. **E,** The relationship between the expression of LINC01554 and disease-free survival in patients with hepatocellular carcinoma was analyzed by the TCGA database. * p <0.05 and ** p <0.01.

tion of HCC cells (Figure 2B, 2C). Meanwhile, it was found that upregulation of LINC01554 reduced the migration and invasion ability of HCC cells, measured by transwell assay (Figure 2D, 2E). The above observations suggest that the ability of cells to grow and metastasize is significantly limited after upregulating LINC01554 in HCC cells.

LINC01554 Could Compete With NGFR to Bind to MicroNRA-3681-3p

Bioinformatics method identified a set of miRNAs that can bind to LINC01554, among which, microRNA-3681-3p was chosen for further research (Figure 3A). Luciferase reporter gene experiment confirmed the binding link between microRNA-3681-3p and LINC01554 (Figure 3B). Furthermore, RIP assays detected that microRNA-3681-3p was physically associated with LINC01554 (Figure 3C). After suppressing LINC01554, microRNA-3681-3p expression was found oppositely enhanced (Figure 3D). Then, the target genes that can bind to microRNA-3681-3p were predicted through bioinformatics, and NGFR was selected as research subject (Figure 3E). Reporter gene assay verified that microRNA-3681-3p bound to NGFR and negatively regulated its expression (Figure 3F). Consistent with this result, qRT-PCR analysis indicated an elevated expression of NGFR induced by microRNA-3681-3p inhibition (Figure 3G). To further verify the regulatory mechanism between the three, LINC01554 and microRNA-3681-3p expressions in HCC cells were simultaneously repressed, and then, NGFR mRNA level was detected by qRT-PCR. The results showed that inhibiting LINC01554 markedly reduced NGFR expression, which was reversed by simultaneous downregulation of LINC01554 and microRNA-3681-3p (Figure 3H). These results indicate that LINC01554 may regulate NGFR expression through binding to microRNA-3681-3p.

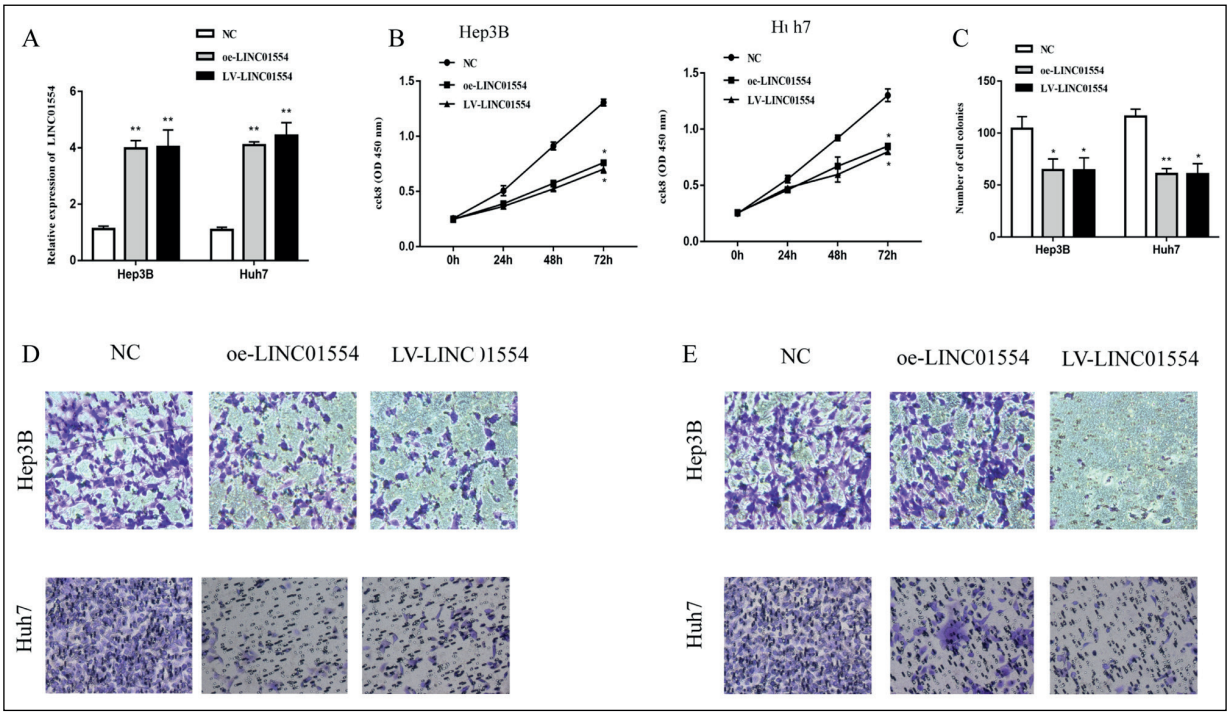

Figure 2. Overexpression of LINC01554 inhibited proliferation, migration, and invasion of hepatocellular carcinoma cells. **A,** Hep3B and Huh7 cell lines were transfected with LINC01554 overexpression plasmid (oe-LINC01554) and lentivirus-encapsulated overexpression plasmid (LV-LINC01554). **B, C,** The CCK8 experiment (**B**) and the cell clone formation experiment (**C**) detected changes in cell proliferation ability after overexpression of LINC01554 in Hep3B and Huh7 cell lines. **D, E**, Transwell migration (**D**) and invasion (**E**) experiments detected changes in cell migration and invasion after overexpression of LINC01554 in Hep3B and Huh7 cell lines (magnification: 20×). * p <0.05 and ** p <0.01.

Downregulation of LINC01554 Expression Partially Reversed the Biological Effects of Overexpression of NGFR

TCGA database revealed that HCC tissues contained a lower expression of NGFR than normal tissues (Figure 4A). Consistently, qRT-PCR detection also revealed the same results in collected 60 pairs of tissue samples (Figure 4B). Next, the impacts of NGFR on cell functions were tested *in vitro*. Overexpressing NGFR remarkably weakened the proliferation ability of HCC cells, while the inhibition of LINC01554 enhanced that (Figure 4C, 4D). Similarly, HCC cell migration ability showed the same tendency (Figure 4E, 4F). These data suggest that LINC01554 may affect the progression of HCC by interacting with microRNA-3681-3p and regulating NGFR expression.

Discussion

The occurrence and development of liver cancer are manipulated by multiple genes and affected by various environmental factors, but its specific mechanism remains unclear. In recent years, abnormal expression of lncRNA has been reported to have great relevance to the development of liver cancer. Therefore, studying the association between the two may provide new ideas for revealing the molecular mechanism of liver cancer and thus developing new therapeutic drugs[14,15]. In the past, LncRNA has not been valued because it does not have the function of encoding proteins. However, with the rapid development of whole gene sequencing technology, more and more lncRNA's roles and functions in liver cancer have been reported. The biological functions of lncRNA in tumor gene expression and transcription, post-transcriptional and epigenetic regulation have been gradually revealed, and its role in liver cancer has gradually received attention[15,16]. LncRNA LINC00662 promotes M2 macrophage polarization and HCC progression by activating Wnt/β-catenin signaling[17]. LncRNA FAM83A-AS1 enhances the stability of FAM83A mRNA by binding to NOP58, thereby accelerating the progression of liver cancer[18].

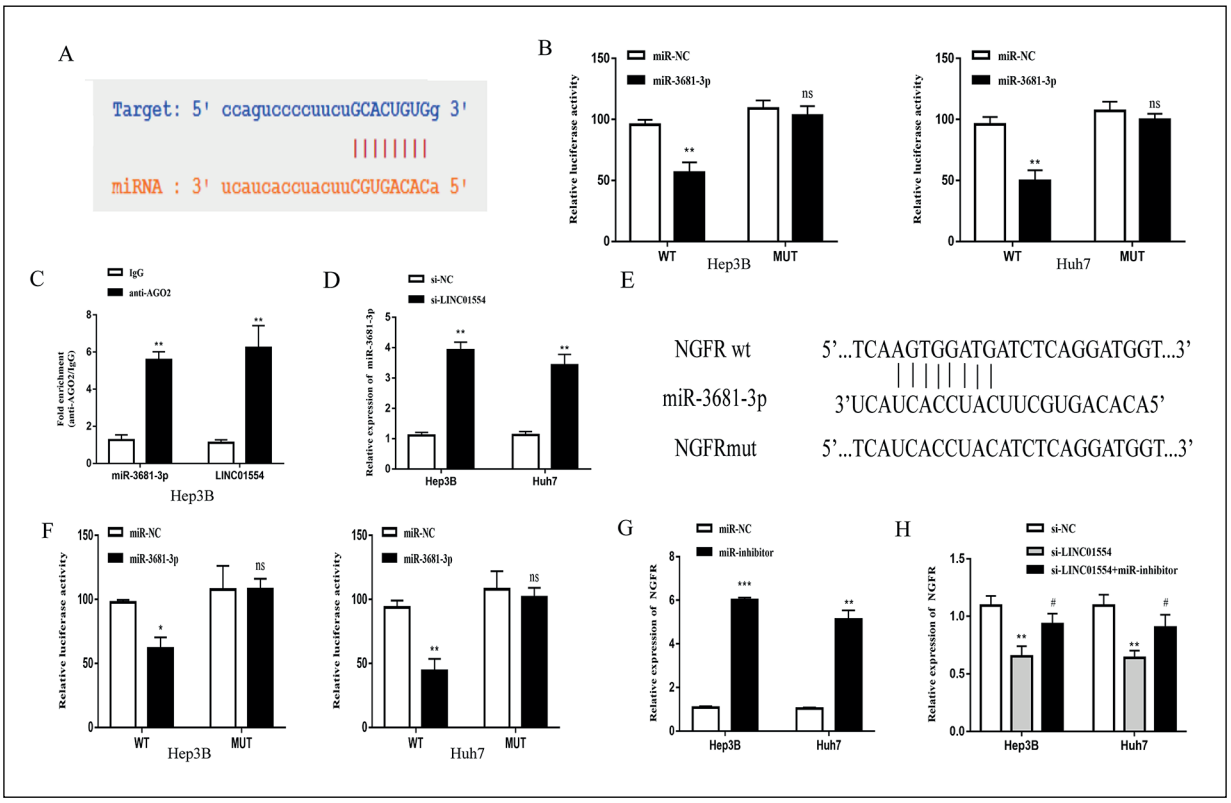

Figure 3. LINC01554 was able to competitively bind to miR-3681-3p with NGFR. **A,** Bioinformatics predicted the binding site of LINC01554 to miR-3681-3p. **B,** The dual luciferase reporter assay was used to detect the binding relationship between miR-3681-3p and LINC01554. **C,** The enrichment of miR-3681-3p and LINC01554 in IgG and AGO2 was detected by RIP experiments in Hep3B cells. **D,** After inhibiting the expression of LINC01554 in Hep3B and Huh7 cell lines, the expression of miR-3681-3p was detected by qRT-PCR. **E,** Bioinformatics predicted the binding sites of NGFR to miR-3681-3p. **F,** Dual luciferase reporter assay was used to detect the binding relationship between miR-3681-3p and NGFR. **G,** After miR-3681-3p was overexpressed in Hep3B and Huh7 cell lines, the expression of NGFR was detected by qRT-PCR. **H**, In Hep3B and Huh7 cell lines, the expression of NGFR was revealed by qRT-PCR after inhibiting the expression of LINC01554 and miR-3681-3p simultaneously. * p <0.05, ** p <0.01, *** p <0.001, ns no significant difference, and # p <0.05.

In this study, it was found that LINC01554 expression was downregulated in HCC tissues and cell lines, and the low expression of LINC01554 heralds a poor prognosis for HCC patients. *In vitro* experiments showed that upregulating LINC01554 remarkably led to reduction in the proliferation rate and migration capacity of HCC cells. With the help of bioinformatics analysis, a potential binding site of LINC01554 on microRNA-3681-3p was found. In addition, this study indicated that LINC01554 directly bound to microRNA-3681-3p and negatively modulated its expression.

Further, it was predicted through the bioinformatics website that LINC01554 might compete with NGFR to bind to microNRA-3681-3p. As a member of the neurotrophic factor family, NGFR is a multifunctional, polypeptide chain-containing substance that binds to nerve growth factor receptors on the surface of corresponding cells to produce corresponding biological effects[19]. Co-receptors of NGFR include tropomyosin-related kinase receptors, which specifically bind to mature neurotrophic factors, and a receptor called sorting protein, which binds specific neurotrophic factor precursors[20,21]. TrkA/NGFR high affinity heterodimer binds to NGF and NT-3, TrkB/NGFR binds to BDNF and NT-4, TrkC/NGFR binds to NT-3, and sorting protein/NGFR complex binds to NGF precursor, NT-3 Precursors and BDNF precursors. In most cases, activation of Trk/NGFR promotes cell survival, while sorting protein/NGFR complex promotes cell death[22,23]. In this study, it was found that NGFR was under-expressed in liver cancer tissues, and the overexpression of NGFR suppressed cell prolifer-

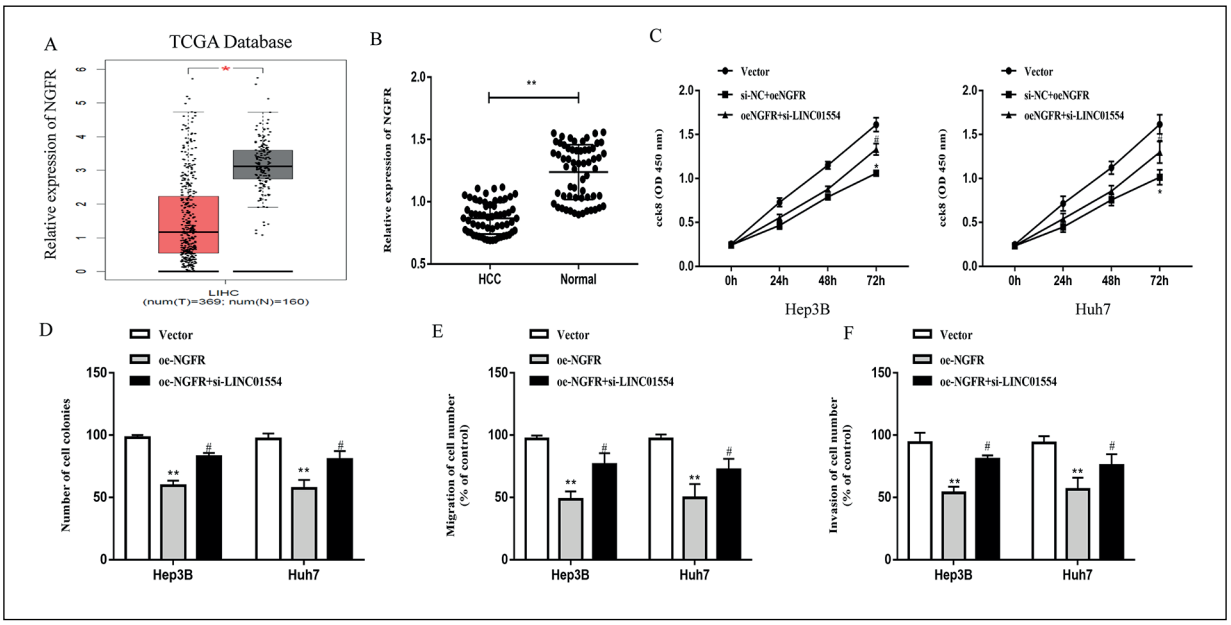

Figure 4. Inhibition of LINC01554 expression partially reversed the biological effects of overexpressing NGFR on cell proliferation. **A,** NGFR expression level in The Cancer Genome Atlas (TCGA) database. **B,** NGFR expression in hepatocellular carcinoma and adjacent normal tissues. **C, D**, After over-expressing NGFR in Hep3B and Huh7 cell lines, the cell proliferation ability was weakened, and the effect was partially reversed after suppression of LINC01554. **E, F,** After over-expressing NGFR in Hep3B and Huh7 cell lines, the cell migration (**E**) and invasion (**F**) capabilities were weakened, and the effect was partially reversed after inhibition of LINC01554 (Magnification: 20×). * p <0.05, ** p <0.01 and # p <0.05.

ation and invasiveness of HCC cells. In addition, microRNA-3681-3p overexpression promoted the degradation of NGFR. Thus, it was suspected that LINC01554 might protect NGFR from degradation by inhibiting microRNA-3681-3p. However, the specific regulation and biological functions still need to be verified in animals. This study initially demonstrated the biological role of the LINC01554/microRNA-3681-3p/NGFR regulatory axis HCC, which can provide a new perspective and theoretical basis for the clinical diagnosis and prognostic treatment of HCC.

Conclusions

These results showed that LINC01554 is remarkably under-expressed in HCC tissues and may serve as a tumor suppressor gene that regulates NGFR expression by binding to microRNA-3681-3p, thus affecting the progression of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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